Nonradioactive, Digoxigenin-labeled DNA Probes for the Detection of Five RNA Species Present in Beet Necrotic Yellow Vein Virus

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Complementary DNA (cDNA) clones corresponding to each of five distinct RNA species of beet necrotic yellow vein virus (BNYVV) were synthesized and identified. The sizes of each cDNA clone for RNAs 1, 2, 3, 4 and 5 molecules were 3.0, 1.7, 1.8, 1.5 and 1.4 kbp, respectively. cDNA inserts to RNA 1 and RNA 2 were covered at a part of the 3' regions, and those of RNAs 3, 4 and 5 were almost full-length. The plasmids containing each of cDNA inserts were labeled with digoxigenin by the random priming method. Northern blot hybridization tests showed that individual probes hybridized specifically to each of the five RNAs. Good results were obtained with 1 to 10 ng of RNA as a mixture of five RNAs, but the probe to RNA 3, RNA 4 or RNA 5 gave a weak signal with heterologous RNAs when more than 10 ng RNA was used. In dot blot hybridization, the limit of detection was about 10 pg RNA, but if a higher content of RNA was spotted, cross reaction occurred using heterologous RNAs. For laboratory and field isolates of BNYVV, each of RNAs 3, 4 and 5 was easily detected by Northern blot hybridization in total nucleic acids extracted from *Tetragonia expansa* leaves inoculated mechanically, but not from roots of sugar-beet plants inoculated by the fungus *Polymyxa betae*. However, satisfactory results were obtained with partially purified or concentrated preparations from roots. These findings indicate that the digoxigenin-labeled probes are useful for the identification and detection of RNAs contained in field and laboratory isolates of BNYVV.

**Key words:** Sugar beet, Rhizomania, BNYVV, RNA detection, Nonradioactive cDNA probe

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INTRODUCTION

Nucleic acid hybridization used widely in molecular biology has been adapted as a tool for the detection of plant and animal viruses. This technique is not only useful for all of the genetic information in the viral nucleic acids, but also for detection and identification of RNA species for epidemiological studies in nature (Miller and Martin 1988). The radioactive labeling ($^{32}$P) system has been most commonly used, but the development of nonradioactive probes has enhanced the applicability of nucleic acid probes as diagnostic tools, because of isotope instability, health hazards, disposal problems and extended time for autoradiography. The use of such hybridization methods for detection of plant viruses has increased recently with digoxigenin-labeled probes (Mas et al. 1993, Dietzgen et al. 1994, Harper and Creamer 1995, Wesley et al. 1996).

Beet necrotic yellow vein virus (BNYVV), responsible for rhizomania disease of sugar beet, is transmitted by the soil-borne fungus *Polymyxa betae*, and usually confined to the roots of sugar-beet plants (Tamada 1975, Richards and Tamada 1992). Although BNYVV has a bipartite plus-stranded RNA genome (RNA 1 and RNA 2), field isolates have two or three satellite-like RNA species, RNA 3, RNA 4 and/or RNA 5 (Bouzoubaa et al. 1985, Koenig et al. 1986, Tamada et al. 1989). Both RNAs 3 and 4 are essential for disease development and spread in nature; RNA 3 causes proliferation of rootlets of sugar beet and facilitates spread of virus in root tissue (Tamada and Abe 1989, Tamada et al. 1990, Koenig et al. 1991), whereas RNA 4 is required for efficient transmission by *P. betae* (Tamada and Abe 1989). BNYVV RNA 5 is present in some isolates in Japan, and is associated with the severity of symptom development (Tamada et al. 1989, Kiguchi et al. 1996, unpublished data). Thus, BNYVV has been known as one of the unique plant viruses containing the smaller RNA species, and therefore it is worthwhile to develop the technique for the detection of such distinct RNA species (RNAs 3, 4 and 5) contained in field and laboratory isolates of BNYVV.

In this paper, we synthesized cDNA clones corresponding to each of five distinct RNA species of BNYVV and describe the use of nonradioactive, digoxigenin-labeled cDNA probes for the specific detection of RNA species in field and laboratory isolates.

MATERIALS AND METHODS

*Virus isolates, propagation and inoculation*
For cDNA cloning, the following laboratory isolates of BNYVV containing each distinct RNA species were used: S-3 (RNA 1+2+3), S-4 (RNA 1+2+4) and D-5 (RNA 1+2+5) (Tamada et al. 1989). These isolates were originally obtained from the S and D field isolates by single lesion transfers in Tetragonia expansa leaves inoculated mechanically. The virus isolates were propagated in inoculated leaves of T. expansa in a glasshouse. Leaf extracts for use as virus inoculum were made in 0.1 M-phosphate buffer, pH 7.4, containing 0.5% 2-mercaptoethanol (Tamada et al. 1989).

Laboratory isolates S-34 (RNA 1+2+3+4), S-3, S-4, D-5, D-6 (RNA 1+2+3+5a) and S-0 (RNA 1+2), and twelve other field isolates were used as samples for RNA detection. The laboratory isolates were propagated in T. expansa leaves (Tamada et al. 1989). The field isolates were inoculated to sugar-beet roots by virus-carrying P. betae, and propagated in special test tubes, filled with quartz sand in the growth cabinet as described by Tamada et al. (1989). Root samples of sugar beet seedlings were used for the RNA detection. On the other hand, these isolates were propagated in inoculated leaves of T. expansa and used as leaf samples for the detection.

**Virus purification and RNA extraction**

Virus particles were purified partially from inoculated leaves of T. expansa and viral RNA was obtained as described by Tamada et al. (1989). Purified virus was dissociated in 2% SDS, 30 mM-sodium phosphate, pH 7.2, 1 mM-Na₂EDTA at 60°C for 5 min. Protein was removed by phenol extraction and RNA was precipitated by ethanol at −80°C for 30 min and pelleted by centrifugation. After being washed with cold 70% ethanol, the RNA was suspended in distilled water. For isolation of RNA 3, the extracted RNA (from isolate S-3) was electrophoresed in agarose tube gels (7 mm × 110 mm) under non-denaturing conditions. A band of RNA 3 was excised from the gels which were stained with ethidium bromide for 1 min. The RNA was recovered by electro-elution, precipitated by ethanol and dissolved in distilled water. For isolation of RNAs 1, 2 and 4 (from isolate S-4) and RNA 5 (from isolate D-5), the RNAs extracted from each of three isolates, were further purified by oligo (dT) cellulose column chromatography into poly(A) + fractions.

**RNA gel electrophoresis**

Viral RNA was analyzed by agarose gel electrophoresis as described by Tamada et al. (1989).

**cDNA synthesis and cloning**
cDNA was synthesized as described by Gubler and Hoffman (1983) using a commercial kit (Amersham). First strand cDNA synthesis reaction was catalyzed by avian myeloblastosis virus reverse transcriptase. The second strand cDNAs were synthesized using DNA polymerase I and RNase H. The double-stranded cDNA was inserted into the HincII site of pUC19 or pUC119. Recombinant plasmids were transformed into competent Escherichia coli NM522. Plasmid DNAs containing cDNAs were isolated from E. coli by the alkaline lysis method as described by Maniatis et al (1982). The size of cDNA inserts was measured by agarose gel electrophoresis after restriction enzyme digestion with XbaI and PstI. For some clones, the origin of cDNA was identified by Northern blot hybridization with digoxigenin-labeled cDNA clones as described below. Some cDNA clones obtained were also examined by the presence of restriction sites (Saito et al. 1996).

**Labeling cDNA with digoxigenin**

Plasmid (3 µg) containing cDNA was labeled with digoxigenin (DIG)-11-dUTP (Boeringer Mannheim) by the random-priming method of Feinberg and Vogelstein (1983).

**Northern blotting**

RNA prepared from purified virus particles or total RNA extracted from healthy or virus-infected tissue was incubated at 55°C for 15 min in 50% formamide and 6% formaldehyde and 20 mM-MOPS (morpholinopropanesulfonic) containing 5 mM-sodium acetate and 0.5 mM-Na₂EDTA, pH 7.0. The RNA samples were electrophoresed on 1.5% agarose gels containing 6% formaldehyde in the same MOPS buffer. After electrophoresis, the gel was washed three times in distilled water and twice in 20 X SSC (3 M-sodium chloride and 0.3 M-sodium citrate) for 10 min each. The RNA was transferred to a nitrocellulose membrane (Advantec Toyo) in 20 X SSC for at least 16 hr, followed by baking for 2 hr at 80°C (Maniatis et al. 1982).

**Dot blotting**

Viral RNA was denatured at 70°C for 10 min, chilled on ice, diluted to the desired concentration and spotted on the nitrocellulose membranes. The spotted membranes were baked at 80°C for 2 hr. One µl of RNA sample was usually spotted on the membranes.

**Hybridization**

For Northern and dot blot hybridization, membranes were prehybridized for 4 hr at 42°C in prehybridization solution containing 50% formamide,
4 X SSC, 40 mM-sodium phosphate, pH 6.5, 1 X Denhardt solution and 200 
μg/ml heat-denatured herring sperm DNA. The prehybridization solution 
was discarded and replaced with fresh buffer to which 50 ng/ml-labeled 
cDNA probe was added. The membranes were incubated at 42°C overnight, 
followed by washing twice for 5 min each time with 2 X SSC containing 0.1%
SDS and then twice at 42°C for 15 min each with 0.1 X SSC containing 0.1%
SDS.

Colorimetric detection

After being washed for 1 min in TN buffer I (0.1 M-Tris-HCl, pH 7.5, 
0.15 M-sodium chloride), the membranes were incubated at 37°C for 30 min 
at a concentration of 150 mU/ml Dig-AP conjugate (polyclonal sheep anti-
digoxigenin-Fab fragments conjugated to alkaline phosphatase) in TN 
buffer I. The membranes were washed three times for 10 min each with TN 
buffer I containing 0.05% Triton X-100, followed by washing for one min 
each in TN buffer II (0.1 M-Tris-HCl, pH 9.5, 0.1 M-sodium chloride, 0.05 
M-magnesium chloride). For color development, the membranes were incu-
bated for 10 to 180 min at room temperature with TN buffer II containing 
3.5 μl BCIP (5-bromo-4-chloro-3 indolyl phosphate) stock solution (50 mg/ml, 
N, N-dimethyl formamide) and 4.5 μl NBT (nitro blue tetrazolium) stock 
solution (75 mg/ml 70 % N-N-dimethyl formamide). The reaction was 
stopped by washing the membranes with distilled water.

Sample preparation

About 50 to 100 mg of leaf or root tissue was grounded into a 1.5 ml 
microtest tube containing 0.4 ml of TS buffer (25 mM-Tris-HCl, pH 7.5, 2%
SDS) with a glass rod, unless otherwise stated. After addition of 0.4 ml of 
water-saturated phenol, the extract was vortexed and centrifuged at 10,000 
rpm for 5 min. The aqueous phase was extracted with an equal volume of 
chloroform: isoamyl alcohol (24 : 1) to remove any residual phenol. The 
nucleic acids were precipitated by adding ethanol to the aqueous phase, 
followed by incubation at −80°C for 30 min (or at −20°C overnight). After 
centrifugation, the nucleic acid pellet was washed with cold 70% ethanol, 
dried under vacuum, resuspended in 0.1 mM-Na₂EDTA (1 μl/mg leaf or root 
tissue) and stored at −20°C.

For another method, viral RNA was prepared from partially purified 
particles by the method described by Tamada et al. (1989). About 0.1 to 1 g 
of leaf or root tissue was ground with a pestle and mortar in 25 ml of 0.5 
M-sodium borate buffer, pH 9.0, containing 1 mM-Na₂EDTA. After a low 
speed centrifugation, the supernatant was added to 2% of Triton X-100,
followed by high speed centrifugation (45,000 rpm, 90 min). The resulting pellet was resuspended in distilled water (usually 0.25 \( \mu l/\text{mg root tissue} \)) and was frozen at \(-20^\circ\text{C}\) until use. Nucleic acid samples for hybridization experiments were prepared by phenol extraction as described above.

RESULTS

Analysis of cDNA clones

The obtained cDNAs ranging from 0.1 to 3.0 kbp in size were inserted into pUC plasmids. From cross hybridization tests, the following numbers of clones were originated from distinct RNA species of BNYVV: 11, 36, 8, 10 and 3 clones were derived from RNAs 1, 2, 3, 4 and 5, respectively. cDNA clones corresponding to RNAs 1, 2 and 4 were all obtained from the S-4 isolate and those to RNAs 3 and 5 were from S-3 and D-5 isolates, respectively.

Recombinant plasmids, pMSD49 (3.0 kbp), pMSD25 (1.7 kbp), pMSC8 (1.8 kbp), pMSD96 (probe 4) and pMSD514 (probe 5).

Fig. 1. Genetic map of the genome of BNYVV and the location of cDNA fragments used as probes for the detection of the RNAs. The boxes indicate the open reading frames. The solid circles = the cap structures at the 5' end; (A)n = the poly(A) tails at the 3' end; and CP = the coat protein. The cloned cDNA inserts used as probes are represented as the thick lines. The vertical bars below the lines indicate the restriction enzyme sites. The following enzymes were used: A = AccI; B = BamH1; E = EcoRI; H = HindII; Ha = HapII; P = PmaCI; S = SacI; Sc = ScaI; Sh = SpkI; Sp = SpeI; X = XbaI.
kbp), pMSD96 (1.5 kbp) and pMSD514 (1.4 kbp) with the largest cDNA inserts to RNAs 1, 2, 3, 4 and 5, respectively, were selected and used for further hybridization experiments as probes.

The location and restriction sites of the cDNA inserts are shown in Fig. 1. The pMSD49 and pMSD25 inserts represents about 45% of RNA 1 and about 35% of RNA 2, respectively, at the 3' terminal regions. Most of restriction sites of these cDNA clones corresponded to those of RNAs 1 and 2 of the French isolate of BNYVV (Bouzoubaa et al. 1986, 1987). The cDNA clones to RNAs 3, 4 and 5 were almost full-length (Fig. 1).

Hybridization conditions

The cDNA fragments pMSD49, pMSD25, pMSC8, pMSD96 and pMSD514, which were labeled with digoxigenin (DIG)-11-dUTP by the random-priming method (Feinberg and Vogelstein 1983), were used as probes 1, 2, 3, 4 and 5, respectively (Fig. 1). To increase the sensitivity and specificity of the probes, several hybridization conditions were examined by Northern blot hybridization. For denaturation of samples, formaldehyde treatment produced a clearer hybridization signal than glyoxal treatment. Temperature of hybridization was better at 42°C than at 55 or 68°C. The sonication of the plasmid probes increased 4 times the signal intensity as compared with non-sonicated probes. The concentration of labeled probes of 50 ng/ml was more appropriate than 10 or 100 ng/ml.

![Northern blot hybridization for the detection of BNYVV RNAs of laboratory isolates containing different RNA components. The virus isolates used were S-34 (lane 1), S-3 (lane 2), S-4 (lane 3), D-5 (lane 4), D-6 (lane 5), S-0 (lane 6) and non-inoculated control sample (lane 7), each of which was hybridized with a mixture of five probes (probes 1 to 5). The RNA was prepared from partially purified virus particles of each BNYVV isolate.](image)
Fig. 3. Northern blot hybridization for the detection of each BNYVV RNA in samples containing a mixture of five RNAs (RNAs 1 to 5). The RNA samples were obtained from a mixture of isolates S-3, S-4 and D-5, which were hybridized with the probe 1 (lane 1), probe 2 (lane 2), probe 3 (lane 3), probe 4 (lane 4), probe 5 (lane 5) or non-labeled probe (lane 6). The RNAs were prepared from partially purified particles.

**Northern blot hybridization tests**

To detect the RNA molecules of BNYVV isolates, Northern blot hybridization tests were done using a mixture of five probes (probes 1 to 5). As shown in Fig. 2, strong signals were detected at positions of the corresponding sizes to RNAs 1, 2, 3, 4 and 5. RNA components and the size of laboratory isolates (S-34, S-3, S-4, D-5, D-6 and S-0) corresponded to those obtained by agarose gel electrophoresis analysis (Tamada et al. 1989). Probes 1, 2, 3, 4 and 5 hybridized to RNA samples containing a mixture of the five RNAs, reacted strongly with RNAs 1, 2, 3, 4 and 5, respectively (Fig. 3), but not react with other RNA molecules or non-inoculated control sample. These results indicate that each RNA species of BNYVV is specifically detected by these individual digoxigenin-labeled cDNA probes. Although the ratio of each RNA species was not exactly estimated, satisfactory signals were obtained with 1 to 10 ng of RNA as a mixture of the five RNA species (Fig. 4).
To test the specificity of the probes in detail, the next experiments were made on three probes (probes 3, 4 and 5) using a much higher amount of RNA. As shown in Fig. 5A, probe 3 gave a weak band at positions of the sizes corresponding to RNA 4 or RNA 5. Similarly, probe 4 and probe 5 also reacted slightly with RNA 3 (Fig. 5A). When 100 ng RNA was hybridized to probe 4 or probe 5, a more broad band was produced, indicating that cross-hybridization occurred between RNA 4 and RNA 5 (data not shown). Thus, each clone gives a strong signal with homologous RNA species, but also may give a weak signal with heterologous RNA species, when more than 10 ng RNA was used.

Sequence identity was found in approximately the 3'-terminal 200 residues of RNAs 3, 4 and 5 (Bouzoubaa et al. 1985, Kiguchi et al. 1996), and therefore we tested using a probe in which the homology region of 3'-terminal was removed. After the pMSC8 fragment was digested by XbaI and PstI, the products were electrophoresed on agarose gels. A band of insert DNA was excised from the gels and recovered by electro-elution. By digestion of this insert DNA with HapII, two clones pMSC8HX and pMSC8HP were obtained (Fig. 1). These clones labeled with digoxigenin were used as probes 3A and 3B, respectively (Fig. 1). Probe 3B, which contained the 3'-terminal region, was 270 nucleotides in length. Probe 3A did not contain any sequence identity.

![Detection of BNYVV RNAs by Northern blot hybridization in different preparations of isolate S-34](image)

A mixture of four probes (probes 1 to 4) was used. A. RNAs were prepared from partially purified particles. Total amounts of RNAs were 10 ng (lane 1), 1 ng (lane 2) and 0.1 ng (lane 3). B. Total RNAs were prepared from crude extract of S-34-inoculated leaves of T. expansa, which were sampled from 1 mg leaf tissue (lane 1), 0.1 mg (lane 2) and 0.01 mg (lane 3).
Fig. 5. Specificity of the probes for the detection of BNYVV RNAs by Northern blot hybridization. The RNA samples containing five RNAs (RNAs 1 to 5) were obtained from a mixture of isolates S-3, S-4 and D-5. The RNAs were prepared from partially purified particles. A. Each of probes 3, 4 and 5 was hybridized with total RNAs of 20 ng (lane 1), 10 ng (lane 2) and 5 ng (lane 3). The arrowheads indicate the homologous signal and the asterisks indicate the cross-hybridized signal. B. Each of probes 3, 3A and 3B was hybridized with total RNAs of 100 ng (lane 1), 10 ng (lane 2) and 1 ng (lane 3). The arrowhead indicates the homologous signal and the asterisk indicates the cross-hybridized signal.

Fig. 5B shows that probe 3A hybridized with only RNA-3 molecule, but not with either RNA 4 or RNA 5 molecule, whereas probe 3B gave strong hybridized signals with either RNA 3 or RNA 4 and/or RNA 5. In addition, probe 3B gave a weak signal at the positions of RNA 1 and RNA 2 (Fig. 5B). Thus, such a cross-hybridization between three RNA species was due to the sequence identity (about 70%) of about 200 nucleotides at the 3'-terminal region.
Dot blot hybridization tests

The first experiments were made to examine the sensitivity of DNA probes in dot blot hybridization. A mixture of four probes (probe 1 to probe 4) was used. Viral RNAs prepared from particles of three isolates (S-34, S-3 and S-0) were spotted at different dilution series onto the nitrocellulose membranes. The results showed that strong signals were obtained by using more than 100 pg RNA of any BNYVV isolates (Fig. 6). No signal was detected for control samples from RNA extracted from TMV (Fig. 6) or water (data not shown).

![Image of dot blot hybridization](image)

Fig. 6. Detection of BNYVV by dot blot hybridization in isolates containing different RNA components. BNYVV isolates S-34 (a), S-3 (b) and S-0 (c), and tobacco mosaic virus (d) were used as samples. The RNA amounts of 10 ng (lane 1), 1 ng (lane 2), 0.1 ng (lane 3) and 0.01 ng (lane 4) were blotted on the membranes and were hybridized with a mixture of four probes (probes 1 to 4).

To test the specificity and sensitivity of the probes in more detail, the next experiments were done using probes 3, 4 and 5. Each RNA was prepared by eluting from gels and was spotted at different dilution series onto the nitrocellulose membranes. As shown in Fig. 7, each probe gave a specific hybridization signal when less more 100 pg RNA (1 ng RNA for RNA 5) was spotted. However, when more than 1 ng RNA (10 ng for RNA 5) was used, cross-reaction occurred, although the signals were weak. The detection limit of RNA was about 10 pg as well as results using a mixture of probes described above. Thus, cross-hybridization occurred with increasing amount of RNA may be due to the sequence identity at the 3' end of these RNA species.
Detection of RNAs 3, 4 and 5 from field isolates of BNYVV

To detect and identify smaller RNA species (RNAs 3, 4 and 5) which may contain in field isolates of BNYVV, hybridization tests were done using probes 3, 4 and 5. The total RNAs were directly extracted from leaves of T. expansa. Samples of 12 field isolates were prepared from those after the fifth transfers of mechanical inoculation to T. expansa leaves.
The hybridization tests showed that five isolates contained RNA 5, but six other isolates did not (Fig. 8). Each probe gave hybridization signals to normal-sized RNA molecules and in addition to smaller molecules in some isolates (Fig. 8), indicating that short forms of RNAs appeared in either RNA 3, RNA 4 or RNA 5, depending on the isolate. Positive hybridization signals in different isolates were seen to range from weak to strong, thereby suggesting different levels of RNA in BNYVV isolates.

![Image of probes 3, 4, and 5](image)

Fig. 8. Detection of RNAs 3, 4 and 5 of BNYVV field isolates by Northern blot hybridization in inoculated T. expansa leaves after fifth serial passages. Each of three probes (probes 3, 4 and 5) was used. Lanes 1 to 12 indicate different BNYVV isolates. Total nucleic acids were extracted from crude extracts of inoculated leaves of T. expansa. The arrowheads indicate the positions of individual normal-sized RNAs.

In the next experiments, Northern blot hybridization was done on RNA samples which were directly extracted from roots of sugar beet. The samples were obtained from rootlets of sugar-beet seedlings, which were grown in special test tubes. The results showed that no or only slight hybridization signals were detected for samples from RNA extracted from roots. Also non-specific signals were sometimes seen in root samples (data not shown).

Our ELISA tests revealed that the virus amount in leaf samples was 10 to 100 times higher than in root samples, and therefore in further experiments, nucleic acids were sampled from preparations concentrated or pu-
rified partially from rootlets of sugar-beet seedlings. Fig. 9 shows that each of the three probes gave a strong hybridization signal and normal-sized RNAs were detected in these field isolates propagated in sugar-beet roots by *P. betae*. No signal was obtained in preparations from healthy control rootlets. Thus, for root samples, satisfactory results for RNA detection were obtained in partially purified preparations.

![Image](image_url)

**Fig. 9.** Detection of RNAs 3, 4 and 5 of BNYVV field isolates by Northern blot hybridization in roots of sugar-beet seedlings inoculated by *P. betae*. Each of three probes (probes 3, 4 and 5) was used. Lanes 1 to 8 indicate different BNYVV field isolates. The RNAs were prepared from partially purified preparations of roots of sugar-beet seedlings. The arrowheads indicate the positions of individual normal-sized RNAs.

**DISCUSSION**

In this paper, we synthesized and cloned cDNAs corresponding to each of five distinct RNA species of BNYVV. These clones obtained were previously analyzed for the sequences of the Japanese isolates S (for RNAs 1, 2, 3 and 4) (Saito *et al.* 1996) and isolate D (for RNA 5) (Kiguchi *et al.* 1996). The cDNA clones were labeled with digoxigenin by the random-priming method, because the nonradioactive probes are attractive for routine diagnostic methods due to their safety, long period of life and short time for detection. The results indicate that BNYVV RNA species contained in laboratory and field isolates are easily detected and identified by Northern blot hybridization using these labeled cDNA probes.

It is of particular interest in the virus ecology that the smaller RNA
species of BNYVV control virus spread and survival in nature (Richards and Tamada 1992). Thus BNYVV RNA 3 and RNA 5 are strongly associated with the development of symptoms in sugar-beet roots (Tamada et al. 1990, unpublished data), whereas RNA 4 is essential for efficient transmission by *P. betae* (Tamada and Abe 1989). In addition, as shown in Fig 8, deletion mutants of these RNA molecules have appeared frequently in laboratory isolates propagated by being inoculated mechanically (Tamada et al. 1989, Richards and Tamada 1992). Thus, it is very important to develop the detection system of such smaller RNA species and their deletion mutants.

We have obtained in preliminary tests (Saito et al. 1988) that digoxigenin labeling is much better than photobiotin labeling, because the latter produces a non-specific background color on the membranes and have lower sensitivity. Although the degree of sensitivity of our system also was not directly compared to that of $^{32}$P-labeled probes, the limit for RNA detection by dot blot hybridization was about 10 pg (Figs. 6, 7). This level of sensitivity is similar or somewhat lower than that reported for other plant viruses and viroids, in which $^{32}$P-labeled or biotin-labeled probes were used (Forster et al. 1985, Habili et al. 1987, Roy et al. 1988, Eweida et al. 1989). Digoxigenin-labeled probes have distinct advantages over radioactive($^{32}$P) labeled probes with regard to their cost, safety and stability. For example, our probes were stable for at least two years at $-20^\circ$C. The time required for colorimetric detection of digoxigenin is much less than the autoradiographic exposure time. Furthermore, digoxigenin labeling and detection methods are not complicated and the reagents can readily be obtained as a commercial kit.

In general, dot blot hybridization was more reliable for rapid and simple detection of RNA than Northern blot hybridization (Baulcombe et al. 1984, Maule et al. 1983). However, our dot blot hybridization was not suitable for the detection of smaller RNA species, because the probes cross-hybridized and also produced false positive signals (Fig. 7). In this respect, DNA probes with the homologous regions at the 3'-terminal of RNAs eliminated should be tested and also the extraction procedures should be improved.

From the reason described above, and for the presence of shortened forms of smaller RNA species, Northern blot hybridization tests were shown to be useful for the detection of BNYVV RNA. In this experiment, a hybridization signal was obtained with RNA samples extracted directly from infected leaf tissue (symptoms appeared), but no or only slight signals or, if any, false-positive signals (non specific signal) produced with the samples extracted from root tissue. This problem was overcome by using RNA samples from partially purified preparation. Indeed, when BNYVV
was transmitted by *P. betae*, the amount of virus in roots is usually small, because the virus is restricted in rootlets or in a part of tap roots. Therefore, we recommend estimation of the virus content in roots by ELISA and then examination for the RNA from partially concentrated or purified preparations of the root tissue. From the degree of concentration, we can roughly estimate the virus content in test samples. Satisfactory results were obtained with 1 to 10 ng RNA, when the samples contained four or five RNA species. This amount of viral RNA corresponds to about 20 to 200 ng of virus (BNYVV contains 5% RNA). Thus, for detection of the RNA in sugar-beet roots, the systems described here are complicated and laborious, and also the presence of non-encapsidated viral RNA in the samples remains a problem. Therefore, more simple and direct methods such as reverse transcription polymerase chain reaction must be developed for the detection of smaller RNA species and their deleted forms present in BNYVV isolates.

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